# MEGAHERTZ-GENERATED FEMTOLITER MICROFLUIDIC DROPLETS FOR SINGLE-MOLECULE-COUNTING IMMUNOASSAY

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## ABSTRACT

This paper describes a microfluidic droplet-based approach enabling the measurement of chemical reactions of individual enzyme molecules and its application to a single-molecule-counting immunoassay. A microfluidic device is used to generate and manipulate <10-fL droplets at rates of up to 1.3 MHz. The femtodroplets produced with this device encapsulate single biomolecular complexes tagged with a reporter enzyme; their small volume enables the fluorescent product of a single enzyme molecule to be detected within 10 minutes of on-chip incubation. Our prototype system is validated by detection of a biomarker for prostate cancer down to a concentration of 46 fM.

**KEYWORDS:** Microfluidics, Fast Droplet Generation, Single Enzyme Analysis, Digital Immunoassay

## INTRODUCTION

Water-in-oil droplets are emerging as a potentially powerful technology to quantitatively study compartmentalized reactions of single enzyme molecules and single cells because the concentration of reaction products or secreted molecules exceeds the detection threshold much more rapidly in small confined volumes than in bulk solution. In order for the product resulting from turnover of a fluorogenic substrate by a single enzyme molecule to be detectable within a few minutes using epifluorescence microscopy, the volume of the reaction chamber has been reduced to less than 100 fL. Ultra-small droplets with volumes ranging from 0.5 fL to 2 pL have been used to detect the activity of single enzyme molecules, but the polydispersity of the emulsions used limited the precision and throughput of these studies.[1] Existing microfluidic devices normally generate highly monodisperse droplets at the pico- to nanoliter scale. In such volumes, several hours of enzymatic activity are required to turn over sufficient substrate for single enzyme molecule detection.[2] Furthermore, maximal droplet generation rates are in the 10 kHz range, limiting high-throughput measurements of fast reactions.

## THEORY

The gold standard immunoassay, ELISA (enzyme-linked immunosorbent assay), enables the detection of biomarkers at concentrations above picomolar ($10^{-12}$ M)[3], but there remains an unmet clinical need for detection of biomarkers of neurodegenerative diseases and cancers that are present in biological fluids at concentrations in the range of $10^{-12}$ -$10^{-16}$ M.[3] One promising approach uses the turnover of a fluorogenic substrate within well-arrays containing single enzyme molecules in an assay mixture as the basis for ultrasensitive digital ELISAs.[4] However, the need for mechanical fabrication of femtoliter wells places inherent limits on the scalability and flexibility of ultrasensitive diagnostic assays, which could be overcome by carrying out experiments in microfluidic droplets. We have developed a method based on a multilayered microfluidic device that enables us to measure the enzymatic activity of single enzyme molecules in a few minutes, a property that we have exploited to construct a bead-based ELISA for the detection of a low-abundance protein biomarker.

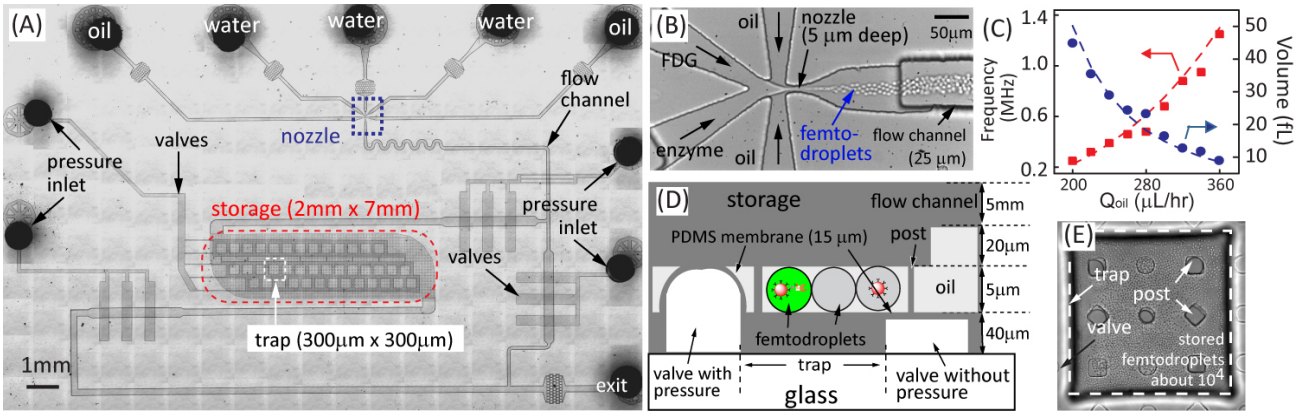


*Figure 1: (A) Photograph of the whole multilayered PDMS device. The upper layer consists of the nozzle, flow channels and storage-area. The bottom layer houses the monolithic valves used to control droplet flow and isolate the traps. (B) Femtodroplet formation at the nozzle of the microfluidic device. Droplets with a typical volume of 32 fL are generated at a frequency of around 3.5 × $10^5$ per second. (C) Generation frequency and volume of femtodroplets as a function of the oil flow rates ($Q_{oil}$) at a constant water flow rate of 40 µL/hr. The dashed line is a prediction curve, fitting the experi-*

*mental data. (D) Vertical schematic of the storage structure. When pressure is applied, the thin PDMS membrane bends up to seal off the flow channel and trap the femtodroplets. (E) Femtodroplets stored in a trap.*

## EXPERIMENTAL

We have developed a microfluidic device for the controlled generation and manipulation of water-in-oil droplets with volumes of 5 - 50 femtoliters - which we call femtodroplets - at frequencies >1 MHz. Microfluidic droplets can be generated by shearing one fluid (water) by a second immiscible one (oil). In order to produce small water droplets at high frequencies, a large shear force and low surface tension at the oil-water interface are required. Large shear forces can be generated by either applying a high flow rate of oil or reducing the channel dimensions in order to increase the flow speed. In order to enhance the flow speed substantially during droplet formation without generating high internal pressure throughout the flow channel in the device, a flow-focusing nozzle was integrated into the design of our device. This strategy introduces a local constriction within a 300-µm section of the device, where the channel dimensions are reduced from 100 × 25 µm (width × depth) to 10 × 5 µm (Figure 1A-B). This nozzle enables the controlled generation of highly monodisperse aqueous droplets in fluorinated oil, previously mixed with a surfactant to decrease the interfacial tension and to prevent coalescence of droplets at frequencies of $10^5$ - $10^6$ Hz (Figure 1C). The femtodroplets formed using our device provide discrete reaction compartments that are small enough to enable the products of one molecule of enzyme to be detected within minutes by epifluorescence microscopy, but also large enough to be manipulated fluidically.

Once single enzyme molecules and the fluorogenic substrate have been encapsulated, it takes a few minutes to accumulate a measurable amount of fluorescent product by a typical reporter enzyme (β-galactosidase).[5] An area of 2 mm × 7 mm (length × width) was therefore integrated into the microfluidic device to store femtodroplets while the enzymatic reaction occurs (Figure 1A). This storage area is divided into 40 traps (300 × 300 µm), isolated by monolithic microfluidic valves (Figure 1D).[6] As the depth of the traps (5 µm) is comparable to the diameter of the femtodroplets, droplets stored in the microfluidic device are packed into a monolayer that allows fluorescence measurements of individual droplets using a simple epifluorescence microscope (Figure 1E).

Trapping the femtodroplets in this way allows the activity of specific enzymes to be monitored continuously inside thousands of droplets simultaneously. The time course of fluorescence generation in approximately 5 × $10^3$ femtodroplets containing 250 µM FDG was imaged at enzyme concentrations of up to 3 × $10^{-2}$ unit/mL (equivalent to about 40 pM), where the likelihood of enzyme occupancy in each droplet is < 0.8 (Figure 2B-C). After incubation for 10 minutes, two populations of droplets were clearly visible (Figure 2A). The fraction of bright femtodroplets followed a Poisson distribution as a function of prepared enzyme concentration, as expected for product formation due to the activity of single molecules of β-galactosidase (Figure 2C-D). The linear relationship between the prepared and determined concentrations of β-galactosidase in Figure 2D confirmed that the activity observed in the bright femtodroplets was due to single enzyme molecules and that a 10-minute incubation in femtodroplet-based digital ELISAs would enable counting of single analyte molecules.

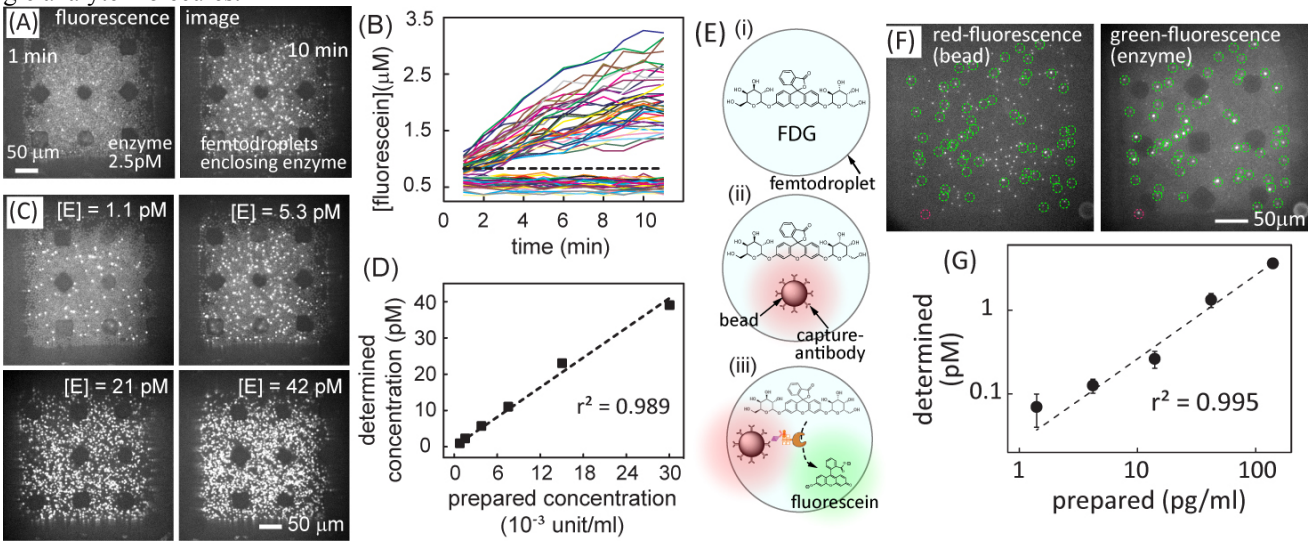


*Figure 2: (A) Images showing green fluorescence resulting from hydrolysis of FDG by β-galactosidase in femtodroplets after 1 and 10 min. The bright spots represent femtodroplets enclosing a single enzyme molecule. (B) Representative time traces of enzyme activity measured in femtodroplets that contain either one β-galactosidase molecule or none. The black dashed line represents a threshold, defined as three standard deviations above the mean of the background fluorescence. The positive traces show a range of activities. (C) Fluorescence micrographs of traps after 10 min incubation at various enzyme concentrations. The fraction of stored femtodroplets that show product formation varies in a concentration-dependent manner. (D) Plot of the prepared concentration vs. the experimentally-determined molar concentration of β-galactosidase. (E) After the on-chip incubation, three populations of femtodroplets are observed, i) droplets containing no bead ii) those containing a bead without immunocomplexes and iii) those containing a bead with an immunocomplex exhibiting a positive fluorescence signal due to the enzymatic activity of a single β-galactosidase reporter.*

*The numerical ratio of (iii) to [(ii) + (iii)] yields the concentration of the target analyte. (F) Red- and green-fluorescence images of stored femtodroplets containing anti-PSA coated beads and substrate after a 10-minute on-chip incubation following immunoassay and subsequent encapsulation. Bright spots in the red-fluorescence micrograph result from beads conjugated with the capture antibody, while those in the green-fluorescence image are due to femtodroplets which contain an enzymatic reporter. The green circles in all images represent droplets that contain a bead and show enzymatic activity. (G) Plot of the molarity of PSA measured by the droplet-based immunoassay vs. the prepared concentration.*

## RESULTS AND DISCUSSION

The ability to sensitively detect β-galactosidase paves the way for ultrasensitive diagnostics using a bead-based ELISA to quantify very low concentrations (0.046 – 4.6 pM) of the biomarker prostate-specific antigen (PSA), reported by a single enzyme. A monoclonal antibody to the target protein was covalently coupled to 1-µm polystyrene beads to enable capture in PBS buffer and subsequent detection of PSA in a sandwich complex containing a detector antibody specifically bound to a β-galactosidase reporter. At the end of each experiment, three different populations of femtodroplets were observed: i) droplets containing no bead; ii) droplets encapsulating a bead but without detectable enzymatic activity and iii) droplets containing a bead and a positive signal in green-fluorescence microscopy corresponding to the presence of active enzyme conjugated to the target protein (Figure 2E-F). Since the concentration of PSA was lower than the bead concentration during anchoring of the target protein to the beads, Poisson statistics dictate that most beads capture either a single enzyme reporter or none.[5] As the bead concentration is known, the fraction of bead-containing femtodroplets that exhibit enzymatic turnover to the total number of beads can be used to calculate the concentration of PSA (Figure 2F-G). The linear relationship obtained between the prepared and experimentally-determined concentrations confirms that this approach can be used to quantify a low-abundance biomarker. The lowest concentration of PSA assayed using our prototype system was 46 fM, an improvement of nearly two orders of magnitude on standard ELISAs.

## CONCLUSION

This paper describes a microfluidic device that is able to generate and manipulate droplets with volumes of 5 - 50 fL at MHz frequencies. This femtoliter microfluidic droplet-based approach enables the measurement of the activity of a single copy of an enzyme and can be exploited to quantify very low-abundance biomarkers by integrating a bead-based immunoassay with direct counting of individual enzyme molecules for developing a highly sensitive diagnostic test. While currently at an early stage, the microfluidic droplet-based platform we have developed has the potential to play a valuable role in the early identification and monitoring of diseases.[7]

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